CRITICAL REVIEW / SYNTHÈSE CRITIQUE

G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins

Stephen S.G. Ferguson, Larry S. Barak, Jie Zhang, and Marc G. Caron

Abstract: G-protein-coupled receptors (GPCRs) represent a large family of proteins that transduce extracellular signals to the interior of cells. Signalling through these receptors rapidly desensitizes primarily as the consequence of receptor phosphorylation, but receptor sequestration and downregulation can also contribute to this process. Two families of serine/threonine kinases, second messenger dependent protein kinases and receptor-specific G-protein-coupled receptor kinases (GRKs), phosphorylate GPCRs and thereby contribute to receptor desensitization. Receptor-specific phosphorylation of GPCRs promotes the binding of cytosolic proteins referred to as arrestins, which function to further uncouple GPCRs from their heterotrimeric G-proteins. To date, the GRK protein family consists of six members, which can be further classified into subgroups according to sequence homology and functional similarities. The arrestin protein family also comprises six members, which are subgrouped on the basis of sequence homology and tissue distribution. While the molecular mechanisms contributing to GPCR desensitization are fairly well characterized, little is known about the mechanism(s) by which GPCR responsiveness is reestablished, other than that receptor sequestration (internalization) might be involved. The goal of the present review is to overview current understanding of the regulation of GPCR responsiveness. In particular, we will review new evidence suggesting a pleiotropic role for GRKs and arrestins in the regulation of GPCR responsiveness. GRK-mediated phosphorylation and arrestin binding are not only involved in the functional uncoupling of GPCRs but they are also intimately involved in promoting GPCR sequestration and as such likely play an important role in mediating the subsequent resensitization of GPCRs.

Key words: G-protein-coupled receptor, G-protein-coupled receptor kinase, arrestin, sequestration, desensitization.

Résumé : Les récepteurs couplés aux protéines G (RCPG) constituent une grande famille de protéines dont la fonction consiste à convertir les signaux extracellulaires en événements intracellulaires. La signalisation véhiculée par ces récepteurs se désensibilise rapidement, principalement par la phosphorylation des récepteurs, bien que la séquestration et la régulation des récepteurs puissent aussi contribuer à ce processus. Deux familles de sérine/thréonine kinases, les protéines kinases dépendantes des seconds messagers et les kinases spécifiques aux récepteurs couplés aux protéines G (KRG) phosphorylent les RCGP et ainsi contribuent à la désensibilisation des récepteurs. La phosphorylation des RCGP spécifique aux récepteurs favorise le couplage des protéines cytosoliques appelées arrestines, dont la fonction est de découpler les RCGP de leur protéines G hétérotrimériques. La famille des protéines KRG est constituée de six membres qui peuvent être répartis en sous-groupes en fonction de l'homologie de séquence et de leurs similarités fonctionnelles. La famille des arrestines comprend aussi six membres divisés en sous-groupes en fonction de l'homologie de séquence et de la répartition tissulaire. Les mécanismes moléculaires contribuant à la désensibilisation des RCGP sont bien caractérisés, mais on possède peu d'information sur le ou les mécanismes participant au rétablissement de la sensibilité des RCGP, si ce n'est que la séquestration (internalisation) des récepteurs peut être un de ces mécanismes. L'objectif du présent travail consiste à réviser

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Abbreviations: GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; β_2AR, β_2-adrenergic receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; α_{2A}AR, α_{2A}-adrenergic receptor; $AT_{1A}R$, angiotensin II type 1A receptor; βARK, β-adrenergic receptor kinase; PIP_2, phosphatidylinositol 4,5,-bisphosphate; mAChR, muscarinic acetylcholine receptor; β_1AR, β_1-adrenergic receptor; BN, bombesin; GRP, gastrin-releasing peptide; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; NK, neurokinin.

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les connaissances actuelles sur la régulation de la sensibilité des RCGP. Nous réviserons plus particulièrement de nouvelles données suggérant un rôle pléiotrope pour les KRG et les arrestines dans la régulation de la sensibilité des RCGP. La fixation des arrestines et la phosphorylation véhiculée par les KRG contribuent non seulement au découplage fonctionnel des RCGP, mais elles participent aussi très étroitement à la séquestration des RCGP et, de ce fait, jouent probablement un rôle important dans la médiation de la resensibilisation des RCGP.

Mots clés : récepteur couplé aux protéines G, kinase des récepteurs couplés aux protéines G, arrestine, séquestration, désensibilisation.
[Traduit par la Rédaction]

Introduction

G-protein-coupled receptors (GPCRs) are found in a large variety of organisms ranging from slime mold and yeast to mammals. GPCRs are integral membrane receptors, containing seven hydrophobic domains, which transduce the information provided by a wide variety of extracellular signals such as light, odour, taste, pheromones, hormones, and neurotransmitters to the interior of cells through their interaction with heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) (Watson and Arkinstall 1994). G-protein α and $\beta\gamma$ subunits in turn modulate the activity of a diverse number of effector systems, resulting in changes in ionic conductance or second messenger levels (Neer 1995), which ultimately initiate the cellular response to GPCR activation.

The activation or signalling cascade of the GPCRs is counteracted in every cell by intrinsic mechanisms that turn off or dampen the agonist-generated signal; this phenomenon is referred to as desensitization. In addition, continued GPCR signalling requires mechanisms by which these receptors become resensitized. The aim of this review is to summarize recent advances in understanding the functional regulation of GPCR responsiveness, and to outline the contribution of two families of proteins, the G-protein-coupled receptor kinases (GRKs) and arrestins, to the delicate balance occurring in cells between mechanisms of desensitization and resensitization.

D sensitization

The exposure of GPCRs to agonists often results in a rapid attenuation in receptor responsiveness. GPCR desensitization involves the contribution of a combination of events: the uncoupling of the receptor from its G-protein as the consequence of receptor phosphorylation, the internalization (sequestration) of plasma membrane associated receptors, and the downregulation of the total cellular complement of receptors as a result of reduced mRNA and protein synthesis as well as increased lysosomal degradation of preexisting receptors (e.g., Doss et al. 1981; Hadcock and Malbon 1988; Bouvier et al. 1988; Hausdorff et al. 1989; Lohse et al. 1990*a*, 1990*b*; Barak et al. 1994). Receptor sequestration, while potentially contributing to GPCR desensitization as the consequence of the loss of plasma membrane associated receptors, is thought primarily to be associated with receptor resensitization. The time frames over which these processes occur vary from seconds to hours, and this has led to the hypothesis that, at least to some extent, each process can be regulated independently of the others. For example, mutations that completely uncouple the β_2-adrenergic receptor (β_2AR) from its G-protein do not necessarily prevent its sequestration (Hausdorff et al. 1990; Campbell et al. 1991). In addition, receptor downregulation can occur independently of sequestration (Campbell et al. 1991; Barak et al. 1994). Nevertheless, there does appear to be some interdependence of these process, as receptor mutants that do not couple, sequester, or downregulate have been made (Cheung et al. 1989; Hausdorff et al. 1991; Barak et al. 1995). The extent of GPCR desensitization varies from complete termination of signalling, as seen in the visual and olfactory systems, to attenuation of agonist potency and maximal responsiveness in other systems (reviewed by Dohlman et al. 1991; Lohse 1993).

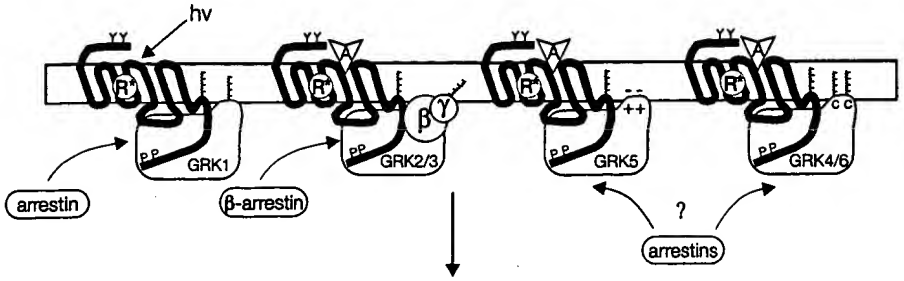
Receptor phosphorylation

Phosphorylation is the most rapid means of GPCR desensitization and is achieved within seconds to minutes of agonist stimulation by two classes of serine/threonine protein kinases: the second messenger activated protein kinases, cAMP-dependent protein kinase (PKA) and protein kinase C (PKC); and the G-protein-coupled receptor kinases (GRKs) that specifically phosphorylate agonist-activated GPCRs (Premont et al. 1995). GRK-mediated phosphorylation of GPCRs serves to promote the binding of arrestin proteins, which when bound further uncouple these receptors (Fig. 1) (Lohse et al. 1990*b*, 1992; Pippig et al. 1993).

Both agonist-dependent and -independent mechanisms of GPCR desensitization have been identified. Agonist-dependent desensitization diminishes a cell's response to only that agonist to which the cell has been exposed and, therefore, is receptor or agonist specific. On the other hand, agonist-independent desensitization of a particular GPCR can occur in the absence of its activation with agonist, as the consequence of the mobilization of second messenger dependent protein kinases in response to the stimulation of another receptor. However, this process is relatively slow, $t_{1/2}$ of 3 min compared with receptor-specific desensitization of GPCRs by GRKs, $t_{1/2}$ of 15 s (Roth et al. 1991).

Both second messenger dependent protein kinases and GRKs contribute to agonist-dependent desensitization of GPCRs. The relative contribution of these two mechanisms to agonist-dependent desensitization is contingent upon agonist concentration, cell type, and the GPCR being studied. For example, mutagenesis and kinase inhibitors have been used to demonstrate that PKA phosphorylation is the most efficient means of desensitizing the β_2AR at low agonist concentrations (nanomolar) (Hausdorff et al. 1989; Lohse et al. 1990*a*). Under these conditions GRK-mediated phosphorylation is negligible, because only a small proportion of receptors are agonist bound. However, since GRK-mediated GPCR desensitization requires agonist activation, this mechanism becomes much more important at high agonist concentrations (Hausdorff et al. 1989; Lohse et al. 1990*a*; Roth et al. 1991). Consequently,

Fig. 1. Schematic representation of the proposed mechanisms of receptor inactivation and membrane targeting by members of the G-protein-coupled receptor kinase family. Agonist activation of GPCRs results in their uncoupling from heterotrimeric G-proteins as the consequence of serine–threonine phosphorylation by GRKs followed by arrestin protein binding. Membrane targeting of members of this family of kinases involves a variety of mechanisms, which include post-translational farnesylation of carboxyl-terminal CAAX motifs (GRK1), interactions with heterotrimeric G-protein βγ-subunits (GRK2 and GRK3), palmitoylation of carboxyl-terminal cysteine residues (GRK4 and GRK 6), and electrostatic interactions with phospholipids as the consequence of a highly basic carboxyl-terminal domain (GRK5). A, agonist; R*, agonist-activated receptor; β and γ, subunits of heterotrimeric G-protein; GRK, G-protein-coupled receptor kinase; wavy lines, farnesyl, isoprenoid, or palmitate groups; *hv*, photon; – –, negatively charged polar phospholipid groups; ++, GRK5 polybasic domain; *c*, cysteine residue; Y, N-linked glycosylation; P, phosphate group.



GRK-mediated mechanisms of desensitization are ideally suited to regulate GPCR function at synaptic locations, where high local levels of neurotransmitters are expected to occur in response to neuronal depolarization. In fact, the predominant expression of GRKs in the brain and highly innervated tissues, as well as their presence in association with postsynaptic densities and presynaptic axon terminals, indicates that they are appropriately positioned to regulate the activity of neurotransmitter receptors (Benovic et al. 1991; Arriza et al. 1992).

In cultured fibroblasts both second messenger dependent kinases and GRKs contribute in a functionally independent manner to the desensitization of the prototypic β_2AR (Hausdorff et al. 1989; Lohse et al. 1990*a*). However, recent studies with other GPCRs suggest that this situation is not quite so simple. Desensitization of the human α_{2A}-adrenergic receptor (α_{2A}AR) (Liggett et al. 1992), rat A_3 adenosine receptor (Palmer et al. 1995), and mouse δ opioid receptor (Pei et al. 1995) appears primarily dependent upon GRK-mediated desensitizing mechanisms, whereas for the rat angiotensin II type 1A receptor (AT_{1A}R), removal of the serine–threonine-rich tail does not affect desensitization (Thomas et al. 1995). The relative contribution of these two kinase families to GPCR desensitization appears even more complex than originally envisaged, since in the olfactory system inhibition of either kinase family results in the complete abolition of olfactory receptor desensitization (Schleicher et al. 1993; Boekhoff et al. 1994). It seems that PKC phosphorylation not only upregulates the activity of GRK2 (Chuang et al. 1995) but targets the kinase to the plasma membrane (Winstel et al. 1996). The importance of GRK-dependent phosphorylation in GPCR desensitization is particularly underscored by experiments with transgenic mice expressing rhodopsin receptors truncated to remove their putative sites of GRK-mediated phosphorylation (Chen et al. 1995). These mice demonstrated abnormally prolonged receptor responses to single photon flashes, attributable to the truncated rhodopsins, clearly demonstrating that GRK-mediated phosphorylation was required for normal shutoff of receptor responsiveness in the visual system.

The G-protein-coupled receptor kinase family

Six distinct cDNAs encoding members of the GRK subfamily of Ser/Thr kinases have been identified and share 53–93% overall sequence homology (Table 1) (Premont et al. 1995). As well, they share similar functional organization. The catalytic domain of these proteins is flanked by an approximately 185 amino acid amino-terminal domain considered to be important for recognition of activated receptor substrate, and a carboxyl-terminal domain demonstrated to be important for membrane targeting (reviewed by Inglese et al. 1993*a*; Lefkowitz 1993; Premont et al. 1995). The members of this family have been classified into subgroups on the basis of sequence homology and functional similarities as follows: (*i*) rhodopsin kinase (GRK1), (*ii*) β-adrenergic receptor kinase 1 (βARK1 or GRK2) and βARK2 (GRK3), and (*iii*) the GRK4 subfamily, comprising GRK4, GRK5, and GRK6 (Premont et al. 1995). The structural and functional aspects of GRKs, as well as their substrate specificity, have been reviewed recently (Inglese et al. 1993*a*; Lefkowitz 1993; Premont et al. 1995; Sterne-Marr and Benovic 1995), thus the present review of GRKs will be limited to those aspects governing their activity and site of action.

GRK targeting

In unstimulated cells, rhodopsin kinase, βARK1, and βARK2 are presumably localized to the cytosol, but their substrate targets are membrane-bound receptors. Therefore, agonist-dependent phosphorylation of GPCRs by these kinases requires their localization at the plasma membrane. For rhodopsin kinase, light-activated association of the enzyme with the plasma membrane is facilitated by post-translational farnesylation of its carboxyl-terminal CAAX motif (Fig. 1) (Inglese et al. 1993*b*). Mutation of the rhodopsin kinase CAAX motif results not only in an enzyme impaired in light-activated translocation to the plasma membrane but one that is substantially less active than the wild-type protein (Inglese et al. 1992, 1993*b*).

βARK1 and βARK2 are not isoprenylated but appear to be

Table 1. Characteristics of GRK family members.

Family name	Size (kDa)	Polypeptide variant (amino acids)	Tissue distribution[a]	Covalent modifications	Activators	References[b]
GRK1 (rhodopsin kinase)	63	nd	Retina > pineal	Farnesylation	Polycations	2, 5, 15
GRK2 (βARK1)	79	nd	*pbl* > *cx* > *h* > *lu* > *k*	nd	βγ-subunits, PIP_2	1, 3, 4, 6, 14
GRK3 (βARK2)	80	nd	*olf* > *b* > *s* > *h* > *lu* > *k*	nd	βγ-subunits, PIP_2	3, 6, 8, 9, 10, 14
GRK4	66	Four	*t* ≫ *b*	Palmitoylation	nd	16
GRK5	68	nd	*h,lu* > *sk* > *b,l* > *k*	nd	Polycations, phospholipids	9, 11, 12, 15
GRK6	66	Yes	*b,sk* ≫ *h,lu,k* > *l*	Palmitoylation	Polycations	7, 13, 15

Note: nd, not determined.

[a]As determined by mRNA expression: *b*, brain; *cx*, cerbral cortex; *h*, heart; *l*, liver; *lu*, lung; *k*, kidney; *olf*, olfactory tuberacle; *pbl*, primary blood leukocytes; *s*, spleen; *sk*, skeletal muscle; *t*, testes.

[b]References: 1, Benovic et al. 1989; 2, Lorenz et al. 1991; 3, Arriza et al. 1992; 4, Chuang et al. 1992; 5, Inglese et al. 1992*b*; 6, Pitcher et al. 1992; 7, Benovic and Gomez 1993; 8, Dawson et al. 1993; 9, Kunapuli and Benovic 1993; 10, Parruti et al. 1993*a*; 11, Kunapuli et al. 1994; 12, Premont et al. 1994; 13, Stoffel et al. 1994; 14, Pitcher et al. 1995; 15, Sterne-Marr and Benovic 1995; 16, Premont et al. 1996.

translocated to the membrane via their interaction with the βγ subunits of heterotrimeric G-proteins (Fig. 1) (Pitcher et al. 1992; Boekhoff et al. 1994). The G-protein γ subunit is geranylgeranylated, and thus translocation of βARK1 and βARK2, at least indirectly, also involves isoprenylation mechanisms (Inglese et al. 1993*a*). The interaction of βARK1 and βARK2 with G-protein βγ subunits involves a βγ binding domain encoded by the 125 amino acid carboxyl-terminal domain of the protein (Koch et al. 1993), which bears striking homology to ~100 amino acid sequences designated as pleckstrin homology domains (Touhara et al. 1994). Effective membrane targeting of βARK1 and βARK2 requires the binding of both βγ subunits and phosphatidylinositol 4,5-bisphosphate (PIP_2) to the carboxyl-terminal pleckstrin homology domain of the kinases (Pitcher et al. 1995). Membrane translocation of βARK can be impaired by polypeptides derived from its carboxyl-terminal domain that function to compete for G-protein βγ subunits (Koch et al. 1993). The inhibition of βARK activity in the hearts of transgenic mice expressing the carboxyl-terminal βγ binding domain of βARK1 has firmly established a role for this domain in regulating the activity of βARKs (Koch et al. 1995).

Unlike GRKs 1–3, GRK4, GRK5, and GRK6 all exhibit substantial membrane localization in the absence of agonist stimulation. Association of GRK5 with membrane is thought to be mediated by electrostatic interaction between its last 46 highly basic carboxyl-terminal amino acids and membrane phospholipids (Fig. 1) (Premont et al. 1994). The activity of this enzyme appears to be dependent upon autophosphorylation of serine and threonine residues, a process that is facilitated by interaction with phospholipids (Kunapuli et al. 1994). In contrast, both GRK4 and GRK6 are palmitoylated at carboxyl-terminal cysteine residues (Fig. 1) (Stoffel et al. 1994; Premont et al. 1996). Palmitoylation of GRK6 seems to be essential for membrane association, as only palmitoylated enzyme was found associated with membrane (Stoffel et al. 1994). GRK4 differs from each of the other identified kinases, because it is the only known GRK with alternative splice variants (Premont et al. 1996). Four distinct alternative splice variants have been identified and are of particular interest since the alternatively spliced exons occur in domains implicated in receptor recognition and membrane targeting (Premont et al. 1996). However, each of the variant forms of GRK4 are significantly associated with membrane and are palmitoylated (Premont et al. 1996).

Site of GRK-mediated phosphorylation

GRKs generally phosphorylate GPCRs at several serine and threonine residues. However, for most GPCRs, little is known about the exact sites of GRK-mediated phosphorylation, and the stoichiometry of phosphorylation differs depending upon the receptor studied and the environment in which it is tested (Haga et al. 1994; Premont et al. 1995). Although GRKs can phosphorylate several sites on receptors in vitro, it is thought that only the initial phosphorylation events are physiologically relevant to receptor desensitization (Ohguro et al. 1993). In fact, high-affinity binding of arrestins to rhodopsin and the β_2AR requires GRK-mediated receptor phosphorylation to a stoichiometry of only 2 moles phosphate per mole of receptor (Gurevich and Benovic 1993; Gurevich et al. 1995).

Although many receptors contain several potential sites for GRK-mediated phosphorylation in their carboxyl-terminal tails, some receptors have shorter carboxyl-terminal tails containing few or no serine and threonine residues, e.g., α_2AR and m2 muscarinic acetylcholine receptor (m2 mAChR). However, these receptors have much longer third intracellular loops and are thought to contain the multiple serine and threonine residues required for GRK-mediated receptor phosphorylation. Consistent with this idea, removal of the serine–threonine-rich portion of the third intracellular loop of the α_2AR reduced agonist-dependent phosphorylation of this receptor by 90% (Liggett et al. 1992). These sites of phosphorylation have been localized to four consecutive serine residues in the third intracellular loop of the α_2AR (Eason et al. 1995). In addition, the sites required for GRK-mediated phosphorylation of the human m2 mAChR have been assigned to serine and threonine residues in its third intracellular loop (Nakata et al. 1994).

Th arrestins

Early studies of rhodopsin and β_2AR receptor desensitization indicated that, although GRK-mediated phosphorylation contributed to receptor desensitization, it was not sufficient for full inactivation under physiological conditions. This indicated

Table 2. Characteristics of arrestin family members.

Family name	Size (amino acids)	Polypeptide variant (amino acids)	Tissue distribution[a]	Substrate	Phosphorylation	Reference[b]
Visual arrestin (S antigen) Bovine	404	396, 370 (p^{44})	*r > pin; cb = pbl* p^{44}: *ros ≫ ris*[c]	Rho > β_2AR > m2 mAChR	PKC Ca^{2+}-calmodulin	1, 2, 4, 6, 9, 10
β-Arrestin1 Rat	418	410	*b > hip > bs,s,o > h ≫ lu,k > pit,sk > l*[d]	β_2AR > m2 mAChR ≫ Rho	?	3, 5, 6, 7, 10
β-Arrestin 2 (arrestin3) Rat	420	409	*s > hip > b > o,bs > pit,h,l ≫ k ≫ lu > sk*	β_2AR, m2 mAChR ≫ Rho	?	5, 7, 10
Cone arrestin (C- or X-arrestin) Human	388	nd	*c > pin > pit,lu*	?	?	8
D-Arrestin Human	?	nd	*l,lu,t,cx > i,h,pit*	?	?	8
E-Arrestin Human	?	Yes	*pit > li,lu,r > h,hyp > cx, i,t*	?	?	8

Note: nd, none detected; ?, unknown.

[a]As determined by mRNA expression: *b*, brain; *bs*, brainstem; *c*, cone photoreceptors; *cb*, cerebellum; *cx*, cerbral cortex; *h*, heart; *hip*, hippocampus; *hyp*, hypothalamus; *i*, intestine; *l*, liver; *lu*, lung; *k*, kidney; *o*, ovary; *pin*, pinealocytes; *pit*, pituitary; *pbl*, primary blood leukocytes; *r*, retina; *ros*, rod outer segment; *ris*, rod inner segment; *s*, spleen; *sk*, skeletal muscle; *t*, testes.

[b]References: 1, Yamaki et al. 1987; 2, Sagi-Eisenberg et al. 1989; 3, Lohse et al. 1990*b*; 4, Yamada et al. 1990; 5, Attramadal et al. 1992; 6, Parruti et al. 1993*b*; 7, Sterne-Marr et al. 1993; 8, Craft et al. 1994; 9, Smith et al. 1994; 10, Gurevich et al. 1995.

[c]As determined by immunofluorescence.

[d]Very abundant in human mononuclear leukocytes.

that some component or "arresting agent" was required in addition to GRK-mediated phosphorylation to quench signal transduction initiated by GPCR activation. The existence of an arresting protein was first demonstrated for photo-excited rhodopsin in rod outer segments in experiments that demonstrated binding of an intrinsic 48-kDa soluble protein, now called arrestin, to phosphorylated rhodopsin (Wilden et al. 1986). The inability of highly purified βARK1 to fully desensitize the β_2AR in vitro, when compared with crude βARK homogenates, suggested that an "arrestin-like" protein was also necessary for appropriate desensitization of the β_2AR (Benovic et al. 1987). The cloning of β-arrestin1, a protein with 59% sequence homology to visual arrestin, confirmed the necessity for a second protein component in β_2AR receptor desensitization (Lohse et al. 1990*b*). Reconstitution of β_2ARs and purified βARK in vitro along with β-arrestin was sufficient to reestablish maximal desensitization of β_2AR activity (Lohse et al. 1990*b*, 1992).

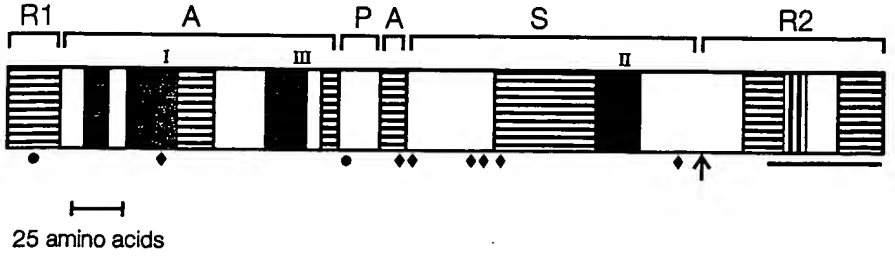
There is now clear evidence for a role of β-arrestin in homologous desensitization in intact cells (Pippig et al. 1993), as well as in the *Drosophila* photosystem in vivo (Dolph et al. 1993). Of particular interest is the observation that β-arrestin1 protein and mRNA levels can be increased by elevated cAMP levels, indicating a novel mechanism for the regulation of GPCR-mediated responses (Parruti et al. 1993*b*). It is now accepted that GPCR desensitization, at least following receptor phosphorylation by GRK1, GRK2, and GRK3, involves the binding of arrestin proteins. However, it is not known whether GRK4, GRK5, or GRK6 target receptors for arrestin binding.

The arrestin family

Arrestins are cytosolic proteins of which six distinct members have now been identified (Table 2). The arrestins can be broken into four subfamilies on the basis of sequence homology and tissue distribution: (*i*) visual arrestin (S antigen), (*ii*) cone arrestin (X-arrestin or C-arrestin), (*iii*) β-arrestins, β-arrestin1 and β-arrestin2 (arrestin 3), and (*iv*) the as yet uncharacterized D- and E-arrestins (Shinohara et al. 1987; Yamaki et al. 1987; Lohse et al. 1990*b*; Attramadal et al. 1992; Murakami et al. 1993; Craft et al. 1994). Vertebrate arrestins share 39–50% sequence homology with invertebrate arrestins, whereas vertebrate arrestins as a family of proteins exhibit 44–84% sequence homology (Craft and Whitmore 1995). Examination of the sequence of the arrestins for known structural motifs has revealed potential consensus phosphorylation sequences as well as possible ATP–GTP binding sites (Fig. 2) (Craft and Whitmore 1995). The role of phosphorylation in the functional regulation of arrestins is unknown. Nonetheless, PKC-mediated phosphorylation of a 48-kDa protein has been detected in bovine rod outer segments, and calcium-dependent phosphorylation of arrestin has been observed in *Drosophila* (Sagi-Eisenberg et al. 1989; Yamada et al. 1990). In addition, ATP binding and hydrolysis by arrestin have also been reported, but there is as yet no indication of what the physiological significance of this finding might be to arrestin function (Glitscher and Rüppel 1991).

Visual arrestin is localized primarily to retina, with low expression in the pineal gland, and is a major protein constituent of rod outer segments (Craft et al. 1994; Smith et al. 1994). Three bovine visual arrestin isoforms have been identified.

Fig. 2. Molecular architecture of arrestins. Horizontal hatched boxes represent arrestin variable regions; closed boxes represent conserved arrestin family domains; and the vertical hatched box indicates region of conservation between the β-arrestins not conserved within the visual arrestin or cone arrestin families (Craft and Whitmore 1995). R1, amino-terminal regulatory domain; R2, carboxyl-terminal regulatory domain; A, activation-recognition domain; P, phosphorylation-recognition domain; S, arrestin hydrophobic domain (Gurevich et al. 1995). Arrow marks position of alternative splicing in the visual arrestin and β-arrestin genes (Parruti et al. 1993*b*; Sterne-Marr et al. 1993). Underlined region highlights the 35 amino acids in visual arrestin replaced by alternative splicing with an alanine to give visual arrestin p^{44} (Smith et al. 1994). Roman numerals represent potential arrestin phosphate binding sites: I, $D(X)_2G$ motif; II, $(A/G)(X)_4GK$ motif, the major consensus features of a phosphate binding site conserved within the visual and β-arrestin subfamilies (Yamada et al. 1990); III, $APQD(X)_2GK$ motif conserved with the visual arrestin family (Yamada et al. 1990; Craft et al. 1995). ◆, potential sites for PKC phosphorylation; ●, potential sites for cGMP-dependent kinase or PKC phosphorylation (Craft et al. 1995).



Bovine visual arrestin is expressed as the originally described 404 amino acid residue form, as well as two polypeptide variants, one in which the last 35 amino acid residues are replaced by an alanine (p^{44}) and the other which lacks residues 338–345 encoded by exon 13 (Fig. 2) (Yamaki et al. 1987, 1990; Parruti et al. 1993*b*; Smith et al. 1994). The p^{44} isoform of visual arrestin has been demonstrated by immunocytochemistry to be localized primarily in the rod outer segment, whereas the distribution of the long form is not limited to the rod outer segment and is present throughout the photoreceptor cell (Smith et al. 1994).

C-arrestin is highly enriched in both retina and the pineal gland, and in situ hybridization studies localize it to cone photoreceptors and to a subset of pinealocytes (Craft et al. 1994). Low levels of C-arrestin mRNA can also be found in the pituitary and cerebral cortex (Craft et al. 1994). No known alternatively spliced variants of this protein have been identified, but C-arrestin is more closely related to the β-arrestins than visual arrestin (Craft and Whitmore 1995). Human C-arrestin maps to the X chromosome, and as such is a candidate gene for cone dystrophy (Craft et al. 1994, Craft and Whitmore 1995). Although no function has been assigned to D- and E-arrestin, these proteins are widely distributed and can be found in a variety of tissues (Craft et al. 1994). D-arrestin expression is not detectable in the hypothalamus, kidney, retina, or spleen, whereas E-arrestin is absent from the kidney and spleen (Craft et al. 1994). E-arrestin has alternative mRNA or tissue-specific forms (Craft et al. 1994).

Similar to visual arrestin, at least two polypeptide variant forms of β-arrestin1 and β-arrestin2 are expressed as the consequence of alternatively spliced mRNAs (Fig. 2) (Sterne-Marr et al. 1993; Parruti et al. 1993*b*). The variant forms of β-arrestin1 differ only by the insertion of eight amino acids between amino acids 333 and 334 (Parruti et al. 1993*b*), and the variant forms of β-arrestin2 (arrestin3) are identical except for an 11 amino acid insert between amino acids 361 and 362 (Sterne-Marr et al. 1993). The existence of alternatively spliced variant β-arrestins increases the number of arrestin homologues that might contribute to the regulation of GPCRs outside the visual system and pineal gland to at least six.

Attramadal et al. (1992) characterized the expression of rat β-arrestin1 and β-arrestin2 and found that they are predominantly localized in neuronal tissues and in spleen, although low mRNA levels can be detected in most rat tissues. Sterne-Marr et al. (1993) demonstrated that either the long or the short form of β-arrestin1 is the most abundant arrestin in all non-photoreceptor-bearing bovine tissues. The long form of β-arrestin1 is the predominant form of β-arrestin1 in the brain, whereas the short form is the major β-arrestin1 in most peripheral tissues except the heart (Sterne-Marr et al. 1993). In contrast, in the rat central nervous system β-arrestin2 is more abundant than β-arrestin1 (Attramadal et al. 1992). Immunohistochemical evaluation of β-arrestin1 and β-arrestin2 expression in the rat brain showed extensive, but heterogeneous, neuronal labelling of the two proteins. β-Arrestins were found in several neuronal pathways, suggesting that they have relatively broad receptor specificity and regulate many GPCRs, not just their namesake, the β_2AR (Attramadal et al. 1992). In addition, immunoelectron microscopy indicated that the β-arrestins were concentrated at synapses along with GRKs, and as such are ideally localized to regulate neuronal activity (Arriza et al. 1992; Attramadal et al. 1992).

Mechanism of action

To quench GPCR signalling, arrestins preferentially bind GRK-phosphorylated receptors, as opposed to either second messenger phosphorylated or nonphosphorylated receptors (Lohse et al. 1990*b*, 1992). For example, GRK-mediated phosphorylation of β_2ARs increases the potency of β-arrestins 10- to 30-fold (Lohse et al. 1992). In in vitro reconstitution experiments with purified components, half-maximal inhibition of β_2AR function was reached at a molar ratio of one or two β-arrestin molecules per GRK-phosphorylated β_2AR, and half-maximal inhibition of rhodopsin activity by visual arrestin was observed at a 1:1 molar ratio (Lohse et al. 1992).

Arrestins are cytoplasmic proteins, yet unlike GRKs, the mechanism(s) by which these proteins are targeted to the membrane to bind and uncouple GPCRs remains unknown. In addition, the regions of GPCRs required for arrestin binding have not been clearly delineated. However, it appears that neither

GRK-mediated phosphorylation nor the cytoplasmic tail of the β_2AR is absolutely required for interaction with either β-arrestin1 or β-arrestin2 (Ferguson et al. 1996). The ability of arrestins to interdict GPCR signalling has suggested that arrestins likely bind intracellular domains implicated in receptor–G-protein coupling. For example, the interaction of visual arrestin with rhodopsin can be inhibited using synthetic peptides representing the first and third intracellular loops of rhodopsin (Krupnick et al. 1994). The dispensability of the cytoplasmic tail for receptor–arrestin binding, at least for the β_2AR, suggests that GRK-mediated phosphorylation of GPCRs either provides secondary binding sites or effects a conformational change in the receptor required for high-affinity β-arrestin binding. Gurevich et al. (1995) examined the ability of several arrestins to bind various functional forms of rhodopsin, the β_2AR, and the m2 mAChR. While each of the arrestins preferentially bound the phosphorylated, agonist-activated (R*-P) form of these receptors, there was still substantial binding to phosphorylated, nonactivated (R-P) receptors, as well as some increased binding to agonist-activated receptor (R*) (Gurevich et al. 1993, 1995). The notable exception was visual arrestin, which clearly preferred phosphorylated, light-activated rhodopsin (Rh*-P).

A kinetic model for arrestin binding to GPCRs has been proposed by the Benovic group (1995) and represents a multistep process that can be summarized as follows (see Fig. 2) (Gurevich and Benovic 1993; Gurevich et al. 1995). First, arrestins are able to bind weakly to any GPCR activation state, allowing the protein to probe the functional status of the receptor. Second, arrestins can bind independently to either GRK-phosphorylated GPCRs (R-P) via the interaction of the arrestin phosphorylation-recognition domain (P) with phosphorylated serine and threonine residues (Kieslbach et al. 1994; Gurevich et al. 1995) or to agonist-activated GPCRs (R*) via the interaction of the arrestin activation-recognition domain (A) with intracellular GPCR domains, including the first and third intracellular loops, which change conformation in response to agonist activation (Krupnick et al. 1994; Gurevich et al. 1995). However, when the receptor is both agonist activated and phosphorylated (R*-P), arrestins bind simultaneously at both sites, inducing a conformational change in the arrestin molecule, serving to present the 120–150 amino acid arrestin hydrophobic domain (S) to the receptor, which not only provides additional binding sites but increases arrestin binding affinity at both primary binding sites (A and P) (Gurevich and Benovic 1993; Gurevich et al. 1995). This change in arrestin conformation leading to the mobilization of the hydrophobic domain is regulated by interactions between the amino (R1) and carboxyl (R2) terminal regulatory domains (Palczewski et al. 1991; Gurevich and Benovic 1993; Gurevich et al. 1994, 1995). This interaction is thought to provide the primary force governing selective binding of arrestins to R*-P by spatially orienting the amino- and carboxyl-terminal halves of the arrestin molecule. The extent of receptor phosphorylation also plays an important role in regulating the affinity of arrestin for Rh*-P, since increased stoichiometry of rhodopsin phosphorylation increases the proportion of bound arrestin (Gurevich and Benovic 1993).

Receptor specificity

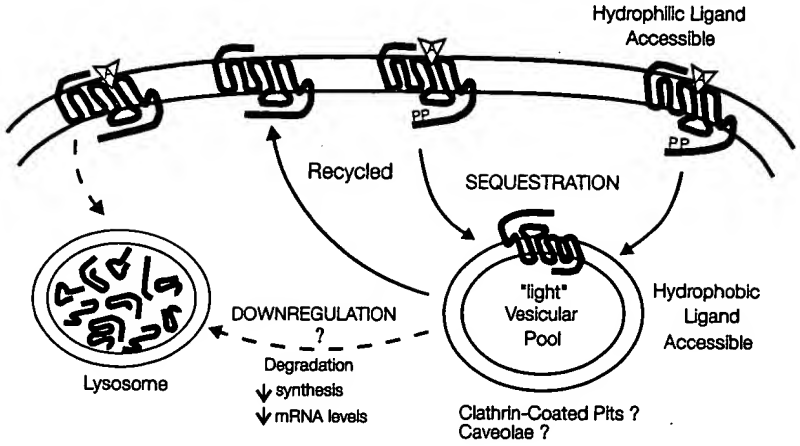
β-Arrestin is 100-fold more effective at uncoupling β_2ARs than visual arrestin, whereas visual arrestin is a much more effective inhibitor of rhodopsin activity than β-arrestin (Lohse et al. 1992). Each of the arrestin subfamilies are most divergent, from one another, at their amino and carboxyl termini, which has led to the suggestion that these regions, in particular the carboxyl-terminal domain, might govern arrestin–receptor specificity. However, since the p^{44} isoform of visual arrestin is a severalfold more potent inhibitor of rhodopsin signal transduction than the long form, the carboxyl-terminal region of the visual arrestin does not appear to be particularly important for directing arrestin binding to rhodopsin (Palczewski et al. 1994). In addition, visual arrestin–β-arrestin1 chimeras, in which the carboxyl-terminal portion of visual arrestin (residues 346–404) has been exchanged for the equivalent residues of β-arrestin1 (341–418), exhibit little change in their relative ability to distinguish between rhodopsin and β_2ARs (Gurevich et al. 1995). Therefore, the regions of visual arrestin governing specificity for rhodopsin as opposed to other GPCRs most likely involve the central 300 amino acid residues and is probably dependent upon the overall tertiary structure of visual arrestin protein.

It is possible that the specificity of visual arrestin binding to rhodopsin might be attributable to differences both in the size of intracellular GPCR arrestin binding domains and the location of relevant GRK-phosphorylated residues. This hypothesis is underscored by the fact that rhodopsin has a relatively short third cytoplasmic loop and all of its GRK phosphorylation sites are contained in the carboxyl tail, whereas the third intracellular loop of the β_2AR is intermediate in size and the third loop of the m2 mAChR is considerably longer and, unlike rhodopsin and the β_2AR, contains putative sites for GRK-mediated phosphorylation. Therefore, it is conceivable that the receptor binding domain of visual arrestin does not tolerate the enhanced steric interference provided by the increased size of the third intracellular loops of either the β_2AR or the m2 mAChR. In addition, this might explain why visual arrestin has severalfold higher affinity for the β_2AR receptor than the m2 mAChR (Gurevich et al. 1995). It is probable that similar considerations govern the GPCR specificity of the other arrestins, since β-arrestin1 binds to the β_2AR with higher affinity than the m2 mAChR, while β-arrestin2 binds to both receptors with equal affinity (Gurevich et al. 1995). Therefore, β-arrestin2 might serve as a "generic" arrestin. As yet, no functional differences have been reported for the variant forms of β-arrestin1 and β-arrestin2. Nonetheless, a definitive answer to the question of arrestin–receptor specificity will have to await further investigation. However, considering the limited number of arrestins and the preponderance of GPCRs, in all likelihood, receptor specificity is governed primarily by tissue-specific expression of the arrestin regulatory proteins. For example, in olfactory epithelium, where only β-arrestin2 is expressed, β-arrestin2-specific polyclonal antibodies can specifically block desensitization of odorant receptors in permeablized epithelium (Dawson et al. 1993). The high degree of specificity observed for visual arrestin–rhodopsin interactions is presumably the consequence of the co-evolutionary divergence of these two proteins in the rod outer segment.

R sensitizati n

The regulation of GPCR responsiveness is a delicate process, requiring a coordinated balance between mechanisms contribut-

Fig. 3. Schematic representation of the intracellular redistribution of GPCRs following agonist activation. Agonist activation of GPCRs results in their internalization to an intracellular compartment, likely endosomes, which can be defined on the basis of the inaccessiblity of this compartment to hydrophilic ligand and low density on a sucrose gradient. The mechanism by which this is achieved is thought to involve either clathrin-coated pits or caveolae. Sequestered GPCRs are subsequently recycled back to the plasma membrane as fully functional receptors. GPCR downregulation is the result of decreased receptor protein and mRNA synthesis, as well as increased receptor degradation as the consequence of either the direct mobilization of plasma membrane receptors to lysosomes or the shunting of a fraction of sequestered receptors to the lysosomal compartment. A, agonist; P, phosphate group.



ing to receptor activation, desensitization, and resensitization. However, very little is understood about the process by which GPCR responsiveness is reestablished. The importance of GPCR resensitization to maintaining normal tissue homeostasis is obvious, since irreversible desensitization would result in the complete abrogation of GPCR signalling. Although it is conceivable that there are situations where the absence of resensitization is desirable, this is certainly not true for most GPCRs. A potential mechanism by which GPCRs are resensitized is the agonist-promoted sequestration (internalization) of cell surface GPCRs to endosomes. While sequestration is likely not important for rhodopsin resensitization (rod outer segments are specialized structures lacking endosomes), it has been implicated in the resensitization of several GPCRs.

Receptor sequestration

GPCR sequestration is an agonist-dependent process which promotes the removal of agonist-activated cell surface receptors from the plasma membrane to a membrane-associated intracellular compartment (Fig. 3). This process has been particularly well characterized for the β_2AR (reviewed by Hausdorff et al. 1992) but has also been observed for several other G-protein-coupled receptors, including but not limited to AT_{1A}Rs, β_1ARs, bombesin (BN) receptors, cholecystokinin (CCK) receptors, dopamine D_2 receptors, endothelin$_A$ receptors, gastrin-releasing peptide (GRP) receptors, gonadotropin-releasing hormone (GnRH) receptors, luteinizing hormone (LH) receptors, m1, m2, m3, and m4 mAChRs, neurokinin (NK) receptors, and neurotensin receptors (Green and Liggett 1994; Hunyady et al. 1994*a*; Kawate and Menon 1994; Slice et al. 1994; Arora et al. 1995; Chun et al. 1995; Roettger et al. 1995; Tseng et al. 1995*a*, 1995*b*; Garland et al. 1996; Goldman et al. 1996; Grady et al. 1995*a*, 1995*b*; Hermans et al. 1996; Itokawa et al. 1996; Koenig and Edwardson 1996). Historically, at least for the β_2AR, this compartment containing sequestered receptors was defined by its low density in a sucrose gradient compared with plasma membranes, as well as its inaccessibility to hydrophilic ligands (Fig. 3). Recent experiments using antibodies directed against the β_2AR, as well as monoclonal antibodies for epitope-tagged recombinant β_2ARs, have confirmed that sequestered β_2ARs are indeed translocated to an intracellular compartment following agonist exposure, probably into endosomes (Fig. 3) (von Zastrow and Kobilka 1992). Similar results have been reported for other GPCRs, such as the ET_AR, GRP receptor, CCK receptor, thyrotropin-releasing hormone receptor, and NK receptors (Ashworth et al. 1995; Chun et al. 1995; Grady et al. 1995*a*, 1995*b*; Roettger et al. 1995; Garland et al. 1996). In the case of the β_2AR, sequestered receptors are not degraded but are recycled back to the plasma membrane as competent signalling receptors (Yu et al. 1993). A similar model was recently proposed for the NK_1 receptor (Garland et al. 1996).

Nonetheless, the precise endocytic mechanism by which GPCR internalization is achieved remains controversial, since β_2AR receptor sequestration has been reported to be effected by both clathrin-coated vesicles and caveolae (Fig. 3) (Raposo et al. 1989; von Zastrow and Kobilka 1992). Receptor sequestration can likely be accomplished by both pathways, but the preferred mechanism of endocytosis utilized by a particular GPCR is probably dependent upon both receptor-specific structural determinants and the cellular environment in which it is expressed (i.e., protein composition) (Rapaso et al. 1989; von Zastrow and Kobilka 1992; Roettger et al. 1995; Goldman et al. 1996; Koenig and Edwardson 1996).

It is uncertain whether receptor sequestration represents the first step in the lysosomal degradation associated with the downregulation of GPCRs following long-term agonist exposure. It is possible that while the majority of sequestered receptors are recycled back to the plasma membrane, a small proportion might be subjected to endosomal sorting and targeted to lysosomes for degradation (Fig. 3) (von Zastrow and Kobilka 1992). However, mutation studies have produced receptor mutants that downregulate normally but do not sequester, in addition to receptor mutants that sequester but do not

downregulate (Campbell et al. 1991; Barak et al. 1994). This suggests that sequestration and downregulation can be mediated by distinct processes (Fig. 3).

Biological role for receptor sequestration

The potential spatial uncoupling of the receptor from its effector system as the consequence of sequestration has led to speculation that receptor sequestration might contribute to GPCR desensitization (Hertel et al. 1990). However, while this might be physiologically relevant in the absence of receptor reserve, considerable evidence now suggests that sequestration, at least for the β_2AR, is not intimately involved in desensitization of the signalling function of the receptor. For example, pharmacological treatments that do not effect GPCR signalling, such as concanavalin A and sucrose, have been used to inhibit receptor sequestration without influencing the ability of the receptor to desensitize (Yu et al. 1993; Pippig et al. 1995). In addition, receptor desensitization due to βARK phosphorylation proceeds at a much faster rate than sequestration ($t_{1/2}$ = 15 s vs. 10 min) (Roth et al. 1991). Thus, under normal conditions, sequestration mostly affects desensitized receptors. This has led to the suggestion that the primary role of GPCR sequestration might be to mediate receptor dephosphorylation and resensitization (Sibley et al. 1986; Yu et al. 1993; Barak et al. 1994; Pippig et al. 1995; Garland et al. 1996). Certainly, receptors isolated from the "light vesicular" or "sequestered" pool of membranes are phosphorylated to a lesser extent than receptors isolated from plasma membranes (Sibley et al. 1986). In addition, high levels of receptor phosphatase activity are associated with sequestered vesicular membranes (Sibley et al. 1986), and GPCR resensitization is inhibited using phosphatase inhibitors (Pippig et al. 1995; Garland et al. 1996). Moreover, pharmacological inhibition of sequestration prevents both the dephosphorylation and resensitization of the β_2AR (Pippig et al. 1995). Recent work has suggested that acidification of GPCRs in endocytotic vesicles might elicit a conformational change in the receptor necessary not only for release of bound ligand but for stimulation of GPCR-directed phosphatase activity (Krueger et al. 1995; Garland et al. 1996). In the case of the β_2AR, the mobilization of receptors back to the cell surface is required to reestablish signalling (Pippig et al. 1995).

Receptor determinants of sequestration

Early experiments mapping regions of the β_2AR important for ligand binding, G-protein coupling, desensitization, and sequestration demonstrated similarities in the structural determinants of the receptor necessary for both G-protein coupling and sequestration (Strader et al. 1987; Cheung et al. 1989). In addition, a rough correlation could be drawn between the coupling efficiency of GPCRs and their ability to sequester (Moro et al. 1994). However, more in-depth characterization of regions required for G-protein coupling and sequestration revealed that, while the cellular mediators of GPCR sequestration and G-proteins likely interact at similar sites on the intracellular face of GPCRs, these processes were functionally distinguishable (Cheung et al. 1990; Hausdorff et al. 1990; Campbell et al. 1991; Lameh et al. 1992; Moro et al. 1993; Barak et al. 1994; Hunyady et al. 1994*a*; Moro et al. 1994). Foremost, in S49 murine lymphoma cell lines, which lack $G_s\alpha$ (*cyc*$^-$) or in which point mutations (*unc*) prevented their G-proteins from interacting with the receptor, β_2AR sequestration in response to agonist stimulation was not impaired (Mahan et al. 1985). In addition, replacement of amino acid residues 222–229 in the amino terminal portion of the third intracellular loop of the β_2AR with the corresponding residues from the m1 mAChR receptor resulted in a receptor mutant that was uncoupled but sequestered normally (Cheung et al. 1990). Furthermore, deletion of residues 267–273 in the carboxyl portion of the third intracellular loop of the β_2AR abolished receptor–G-protein coupling without affecting high-affinity agonist binding and receptor sequestration (Hausdorff et al. 1990). In the case of mAChRs, determinants for agonist-promoted sequestration were localized to a small serine–threonine-rich domain in the middle of the third intracellular loop (Lameh et al. 1992; Moro et al. 1993). This observation suggested that receptor phosphorylation may in fact play an essential role for mAChR sequestration (see below).

In addition to the third intracellular loop of GPCRs, other intracellular domains, such as the second intracellular loop and carboxyl-terminal tail, are implicated as important determinants for GPCR internalization. In particular, the highly conserved DRYXXV/IXXPL sequence, found in the second intracellular loop of GPCRs, affects GPCR internalization. Mutation of the leucine residue in this conserved sequence in either the m1 mAChR or the GnRH receptor impairs sequestration (Moro et al. 1994; Arora et al. 1995). In addition, mutation of serine 140 to a tyrosine residue in the second intracellular loop of the GnRH receptor increases the relative sequestration of the GnRH receptor by 60% (Arora et al. 1995). This increase in receptor sequestration is associated with an increase in agonist binding affinity (Arora et al. 1995).

For several receptors, sequestration is impaired following the removal of their carboxyl-terminal tails (e.g., GnRH receptor, α_{1B}AR, parathyroid hormone, and parathyroid hormone related peptide receptor, AT_{1A}R, BN receptor, and neurotensin receptor) (Benya et al. 1993; Lattion et al. 1994; Huang et al. 1995; Thomas et al. 1995; Tseng et al. 1995*b*; Hermans et al. 1996), whereas the sequestration of the m1 mAChR is not impaired by the truncation of its carboxyl-terminal tail, and truncation of the avian β_1AR carboxyl tail promotes its sequestration (Lameh et al. 1992; Parker et al. 1995). In addition, truncated β_2ARs either internalize normally in response to agonist stimulation or are slightly impaired in their sequestration, depending on the cell type tested (Strader et al. 1987; Bouvier et al. 1988; Cheung et al. 1989; Ferguson et al. 1996).

Recently, β_3/β_2AR chimeras were utilized to delineate molecular and structural receptor determinants involved in β_2AR desensitization and sequestration (Jockers et al. 1996). The β_3AR is a particularly useful tool for these studies as a result of its unique inability to either desensitize or sequester (Liggett et al. 1993). Substitution of the first and second loops of the β_2AR into the β_3AR results in a chimeric receptor exhibiting a β_2AR sequestration phenotype, whereas substitution of the β_3AR third intracellular loop with the third intracellular loop of the β_2AR along with any other intracellular receptor domain (e.g., first and (or) second intracellular loop of the β_2AR or the β_2AR carboxyl tail) results in a chimeric receptor that does not internalize. These results are complemented by previous work demonstrating that substitution of the carboxyl-terminal tail of the β_2AR into the β_3AR receptor results in a chimeric receptor capable of sequestering in response to agonist stimulation

(Liggett et al. 1993). These data taken together indicate that agonist-promoted GPCR sequestration requires appropriate interaction of some intracellular component with several intracellular receptor domains, which are important for both G-protein coupling and arrestin binding.

Recent studies have suggested that receptor sequestration might be directed by sequestration-specific sequence motifs (Barak et al. 1994; Hunyady et al. 1994*b*; Huang et al. 1995). Among the most conserved regions of GPCRs is a sequence of amino acid residues, the $NP(X)_{2,3}Y$ motif, found toward the cytoplasmic face of the putative seventh transmembrane domain. This sequence, found in most GPCRs, bears striking resemblance to the NPXY internalization motif described for the low density lipoprotein (LDL) and insulin receptors (Chen et al. 1990; Rajagopalan et al. 1991). Indeed, mutation of the tyrosine residue (Y326A) in the β_2AR results in a receptor impaired in its ability to sequester (Barak et al. 1994). Nonetheless, it is not likely that this sequence represents a common sequestration motif for GPCRs, since mutation of the corresponding tyrosine residues in both the $AT_{1A}R$ and GRP receptor did not impair their sequestration (Slice et al. 1994; Hunyady et al. 1995). Further characterization of this sequence suggests that, while it presumably does not function as a sequestration motif per se, it is a motif that functions as a critical determinant of receptor conformation required for normal interactions with both agonist and G-protein (Barak et al. 1995). As such, it is probable that the $NP(X)_{2,3}Y$ motif plays an important role in the isomerization of GPCRs from their low- to high-affinity conformation ($R \rightarrow R^*$) in response to agonist. This suggests that GPCR sequestration not only requires interaction of some cellular component with multiple intracellular receptor domains but requires a change in receptor conformation associated with agonist activation. This idea is supported by experiments demonstrating that m4 mAChR internalization is regulated by structural determinants required for high-affinity agonist binding but not necessarily G-protein–receptor interactions (Van Koppen et al. 1994), as well as the observation that increased sequestration of the GnRH receptor is associated with increased agonist affinity (Arora et al. 1995).

Molecular determinants of sequestration: GRKs and β-arrestins

Sibley et al. (1986) were the first to suggest that phosphorylation might not only function to uncouple GPCRs but might provide the signal leading to receptor sequestration (Sibley et al. 1986). The dependence of mAChR subtype sequestration upon a series of serine and threonine residues contained within their third intracellular loops supported this idea (Lameh et al. 1992; Moro et al. 1993). However, mutation of all the putative sites for PKA- and GRK-mediated phosphorylation of the β_2AR resulted in receptor mutants that sequestered normally in response to agonist (Bouvier et al. 1988; Hausdorff et al. 1989). These results were corroborated using permeablized A431 cells and inhibitors of PKA and βARK phosphorylation (Lohse et al. 1990*a*) and has led to the commonly held view that receptor phosphorylation is not a prerequisite for GPCR sequestration.

Despite evidence to the contrary, recent studies of the regulation of GPCR responsiveness have renewed interest in a potential role for receptor phosphorylation in agonist-promoted sequestration. The first indication that GRK-mediated phosphorylation facilitates GPCR sequestration came from experiments demonstrating that βARK1 overexpression enhanced m2 mAChR sequestration (Tsuga et al. 1994). In addition, overexpression of a dominant–negative βARK1 (K220W) retarded the internalization of the m2 mAChR receptor when expressed in COS7 cells (Tsuga et al. 1994). However, the effectiveness of dominant–negative βARK1 was dependent upon the cell type in which it was tested, since m2 mAChR sequestration was not inhibited in either BHK-21 cells or HEK293 (Tsuga et al. 1994; Pals-Rylaarsdam et al. 1995).

The demonstration that β_2AR sequestration proceeded in the absence of βARK phosphorylation sites suggested that βARK-mediated facilitation of m2 mAChR sequestration might be unique to this class of G_i-coupled receptors (Tsuga et al. 1994). However, with the use of the sequestration-defective β_2AR-Y326A mutant (Barak et al. 1994), a role for GRK-mediated phosphorylation could also be demonstrated for the G_s-coupled β_2AR (Ferguson et al. 1995). In addition to the sequestration defect, the β_2AR-Y326A mutant did not serve as a substrate for GRK-mediated phosphorylation (Ferguson et al. 1995). However, βARK1, when overexpressed, not only rescued the phosphorylation deficit of the β_2AR-Y326A mutant but rescued its sequestration as well (Ferguson et al. 1995). In addition, rescued β_2AR-Y326A mutant sequestration was entirely dependent upon intact sites for GRK-mediated phosphorylation, and was independent of PKA phosphorylation (Ferguson et al. 1995). Expression of an isoprenylated βARK1 dominant–negative construct (C-20 βARK1-K220M) also impaired both the phosphorylation and sequestration of the wild-type β_2AR (Ferguson et al. 1995). However, the maximal extent of this inhibitory effect was not overwhelming and was not seen using a βARK1-K220R dominant–negative mutant (Kong et al. 1994). Recently, it was shown that β_2AR-Y326A mutant sequestration could be rescued by the overexpression of any GRK exhibiting the capacity to phosphorylate this receptor mutant (Ménard et al. 1996). A role for GRK-mediated phosphorylation was also recently reported for D_2 dopamine receptor sequestration (Itokawa et al. 1996). In addition, it is possible that phosphorylation might play an important role in the sequestration of a wide variety of GPCRs, since removal of the serine–threonine-rich carboxyl-terminal tails of several GPCRs impairs their internalization (Benya et al. 1993; Lattion et al. 1994; Huang et al. 1995; Thomas et al. 1995; Tseng et al. 1995*b*; Hermans et al. 1996).

The observation that β_2ARs lacking putative sites for GRK-mediated phosphorylation can sequester in response to agonist stimulation clearly indicates that GRK-mediated phosphorylation is not absolutely required as a signal initiating sequestration (Hausdorff et al. 1989; Ferguson et al. 1995). Rather, it suggests that phosphorylation either stabilizes the conformation of the receptor required for sequestration or promotes the interaction of GPCRs with some other cellular element that can directly promote receptor sequestration, even in the absence of phosphorylation. Recently, the cellular elements directing β_2AR sequestration were shown to be the β-arrestins, which bind GPCRs in response to GRK-mediated phosphorylation (Ferguson et al. 1996). When overexpressed, β-arrestins rescue β_2AR-Y326A mutant sequestration even in the absence of phosphorylation and, unlike βARK1, promote the sequestration of receptor mutants lacking either their carboxyl-terminal tails or their putative sites for GRK phosphorylation (Ferguson

endocytic mechanism utilized by a given GPCR is likely dependent upon receptor-specific structural determinants as well as the cell type in which the receptor is expressed. A clear answer to this hypothesis will require the study of several different GPCRs, coupled to diverse G-proteins, expressed in a variety of different cell types. Most importantly, identification of other components involved in GPCR endocytosis, in particular those that associate with β-arrestins, will help provide a clearer understanding of the molecular mechanisms involved in receptor-mediated endocytosis and which contribute to the regulation of GPCR responsiveness.

Although the contributions of GRKs and arrestins to the biochemical regulation of GPCR responsiveness are becoming fairly well understood at the level of the single cell, little is known about the physiological contribution of these proteins to tissue homeostasis. In particular, although GRKs and arrestins are discretely localized to synaptic terminals and postsynaptic densities, there is limited or no understanding of their contribution to synaptic transmission. Are these proteins involved in the establishment of memory and learning, by way of modulating neurotransmitter sensitivity? Are changes in their expression levels associated with subtle changes in the biochemistry of the brain related to psychiatric disorders? Are they involved in either the adaptive changes or the toxicities associated with prolonged drug treatment? Are they associated with human pathologies? For example, the demonstration that GRK expression levels are elevated in failing human heart (Ungerer et al. 1993, 1994) and the locus coeruleus of rats chemically treated with morphine (Terwilliger et al. 1994) suggests that this might be an interesting avenue to investigate.

Recently, mutations leading to the constitutive activation of GPCRs have been linked to a variety of genetic diseases (e.g., Parma et al. 1993; Robbins et al. 1993; Shenker et al. 1993). Analysis of several constitutively activated receptors now suggests that these receptor mutants might be constitutively desensitized as the consequence of persistent phosphorylation and arrestin binding (Ren et al. 1993; Pei et al. 1994; Li et al. 1995; Rim and Oprian 1995). Therefore, given the complexity of the interplay between mechanisms contributing to the activation, desensitization, and resensitization of GPCRs, a clearer knowledge of the relative contribution of each of these components in the regulation of GPCR responsiveness will be required. Until then, it is unlikely that an appropriate understanding of the physiological basis of human pathologies associated with GPCRs or the effects of long-term drug treatment can be obtained.

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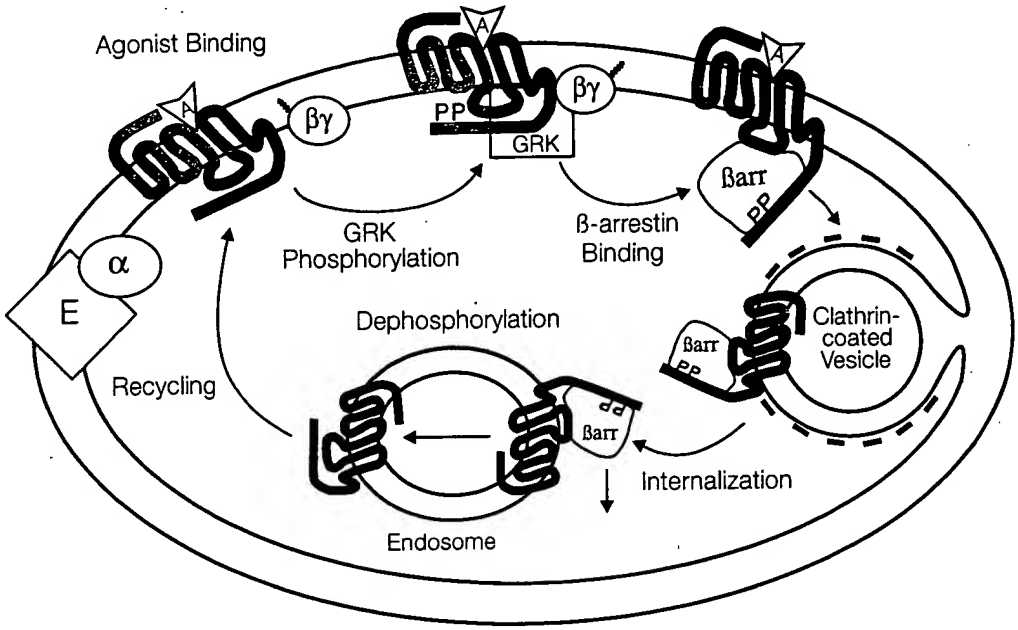
Fig. 4. Schematic representation of GPCR desensitization and resensitization following agonist activation. A, agonist; α, β, and γ, subunits of heterotrimeric G-protein; E, effector enzyme; GRK, G-protein-coupled receptor kinase; βarr, β-arrestin; P, phosphate. See text for details.



et al. 1996). The primary role of GRK-mediated phosphorylation is to increase the affinity of the β_2AR to bind arrestins, since β-arrestin-dependent rescue of β_2AR-Y326A mutant sequestration is potentiated by coexpression of low levels of βARK1 protein (Ferguson et al. 1996). In addition, β-arrestin mutants can be prepared that function as sequestration-specific dominant–negatives (β-arrestin1-V53D and β-arrestin2-V54D) (Ferguson et al. 1996). In agreement with data previously published by Hausdorff et al. (1989), GRK-mediated phosphorylation itself is not sufficient to rescue β_2AR-Y326A sequestration, since overexpression of βARK1 in conjunction with a dominant–negative β-arrestin results in rescued phosphorylation without rescued sequestration (Ferguson et al. 1996). Therefore, β-arrestin proteins play a dual role in the regulation of GPCR responsiveness: they not only bind and uncouple GRK-phosphorylated receptors but they participate in promoting GPCR sequestration. β-Arrestins appear to act as adaptor-like proteins in GPCR trafficking and either serve to recruit other cellular proteins that participate in the mobilization of receptors to endocytotic organelles or execute this function themselves. Potentially, a similar mechanism might be involved in the GRK-facilitated sequestration of the m2 mAChR and D_2 dopamine receptors (Tsuga et al. 1994; Itokawa et al. 1996).

Taken together, the data presented above suggest a model for the regulation of GPCR responsiveness (Fig. 4). Agonist activation of a GPCR results in its isomerization from an inactive conformation to an activated conformation ($R \rightarrow R^*$), which likely requires a conformational change in the receptor mediated, at least in part, by the $NP(X)_{2,3}Y$ motif. This leads to receptor–G-protein coupling, the exchange of GDP for GTP on the G-protein α subunit, culminating in the dissociation of G_α from $G_{\beta\gamma}$ and ultimately activation of an effector system. The agonist-activated receptor can then serve as a substrate for GRK-mediated phosphorylation, which in the case of GRK2 and GRK3, requires $G_{\beta\gamma}$-mediated translocation to the plasma membrane. GRK-mediated phosphorylation of GPCRs promotes the binding of cytosolic arrestin proteins, which when bound, can promote the mobilization of GPCRs for clathrin-coated vesicle-mediated endocytosis (Zhang et al. 1996). Subsequently, GPCRs are translocated to endosomes, where they become dephosphorylated and resensitized. Resensitization of GPCRs requires acidification in endosomes both to release bound ligand, at least in the case of peptide receptors, and to facilitate their dephosphorylation. Although not tested, it is possible that acidification might promote the release of receptor-bound β-arrestin, allowing the interaction of protein phosphatases with the receptor, in a manner similar to arrestin-regulated dephosphorylation of rhodopsin in the eye (Palczewski et al. 1989). Subsequently, GPCRs are mobilized back to the plasma membrane as competent receptors by mechanisms that have not yet been delineated.

Perspectives

The present review has highlighted the contribution of the GRK and arrestin families of proteins to the regulation of GPCR responsiveness. It is now clear that the signals and molecular intermediates culminating in the termination of GPCR-mediated signals can also initiate the events leading to the reestablishment of tissue responsiveness to these signals, receptor sequestration, and resensitization. Of particular interest is the novel role for β-arrestins acting as GPCR trafficking proteins, with respect to the phenomenom of receptor sequestration and resensitization. Given the multiplicity of GPCRs, it is unlikely that β-arrestins are required for the internalization of all GPCRs, as several distinct pathways have been reported to govern GPCR internalization. However, it is likely that β-arrestins play a major role in the internalization of several GPCRs, potentially by specifically directing them to clathrin-coated pits for endocytosis (Zhang et al. 1996). The particular

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